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Hemorrhagic Shock-Induced Vascular Hyporeactivity in the Rat: Relationship to Gene Expression of Nitric Oxide Synthase, Endothelin-1, and Select Cytokines in Corresponding Organs

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Background. **Our previous work observed that vascular hyporeactivity to norepinephrine (NE) developed after hemorrhage and the response was not the same in the 4 arteries examined. To evaluate possible mechanisms involved, the present study investigated the gene expression of iNOS, eNOS, IL-1β, IL-6, TNF-α, and endothelin-1 in the corresponding organs, and the roles of nitric oxide (NO) and endothelin (ET).**

Materials and methods. **LAnesthetized rats (n = 7/time point/group) were hemorrhaged to a mean arterial pressure of 50 mmHg for 60 min. The vascular reactivity of the superior mesenteric (SMA), celiac (CA), left renal (LRA), and left femoral arteries (LFA) to NE was measured at baseline, at the end of the hypotensive period (E), and at 1, 2, and 4 h later in the three groups (hemorrhage, hemorrhage+NG-nitro-L-arginine methyl ester (L-NAME), an NO synthase inhibitor, or hemorrhage+PD142893, an ET receptor antagonist). Gene expression in ileum, left kidney, liver, and skeletal muscle was determined by quantitative RT-PCR at these times.**

Results. **Vascular reactivity of SMA, CA, LRA, and LFA to NE decreased as much as 98% over 4 h compared with baseline. This loss of responsiveness in CA and LFA was more severe than in SMA and LRA. Gene expression of iNOS, eNOS, IL-1β, IL-6, TNF-α, and endothelin-1 in the corresponding organs of select vasculatures increased markedly over baseline levels and the fold increase in mRNA levels of these enzymes and mediators in liver and skeletal muscle was higher than in ileum and left kidney. For example, at 4 h, iNOS expression was over 16-fold higher than baseline in liver and skeletal muscle, but 5- and 7-fold higher in ileum and kidney, respectively. L-NAME or PD142893 partially attenuated the decreased vascular reactivity induced by hemorrhagic shock and attenuated the changes in gene expression observed.**

Conclusion. **These findings suggest that the differential expression of NOS, cytokines, and endothelin-1 in different organs are associated with the development of vascular hyporeactivity after hemorrhagic shock and may account, at least in part, for the vascular bed diversity observed.**

Key Words: **hemorrhage; vascular reactivity; cytokines; nitric oxide synthase; endothelin.**

INTRODUCTION

Studies have shown that the vascular reactivity to vasoconstrictors and vasodilators can be reduced greatly after severe trauma or shock [1–7]. Many factors, including desensitized adrenoceptors [1, 8], nitric oxide (NO) [3, 7, 9–13], endogenous opioid peptides [4, 14], inflammatory cytokines such as TNFα [15, 16] and IL-1 [17] have been proposed to be involved in the development of vascular hyporeactivity during shock. This vascular hyporeactivity may also play an important role in the development and the outcome of the shock state, and can interfere with the therapy of shock by reducing the effectiveness of vasoactive agents [1]. It has been suggested that the low- or non-response of many patients to some vasoactive agents in the late stage of critical disease may be related to vascular hyporeactivity [1]. Consequently, it is very important to elucidate the mechanism responsible for this vascular hyporeactivity and the role of modulating factors.

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TABLE 1

Aspects of the RT-PCR Reaction

Primer Pairs for the Selected Genes

	Sense	Antisense	Product length (bp)
G3PDH	5′-TCCTGCACCACCAACTGCTTAG-3′	5′-TGCTTCACCACCTTCTTGATGTC-3′	341
iNOS	5′-TAGAAACAACAGGAACCTACCA-3′	5′-ACAGGGGTGATGCTCCCGGACA-3′	907
eNOS	5′-CTGCTGCCCGAGATATCTTC-3′	5′-CAGGTACTGCAGTCCCTCCT-3′	228
ET-1	5′-TCTTCTCTCTGCTGTTTGTGGCTT-3′	5′-TCTTTTACGGCTTTCTGCATGGAT-3′	407
IL-1β	5′-GAAGCTGTGGCAGCTACCTATGTCT-3′	5′-CTCTGTTGAGAGGTGCTGATGTAC-3′	520
IL-6	5′-GATGTTGTTGACAGCCACTGC-3′	5′-CACTCCTTCTGTGACTCTAAC-3′	501
TNF-α	5′-TACTGAACTTCGGGGTGATTGTCC-3′	5′-CGTAGGACCCGATGTGACTC-3′	295

PCR condition

	GAPDH	iNOS	eNOS	ET-1	IL-1β	IL-6	TNF-α
Annealing temperature (°C)	57	58	58	55	56	56	57
Elongation temperature (°C)	72	72	72	72	72	74	72
Elongation time (s)	14	30	10	16	20	20	12

Our previous work has shown that the degree of vascular hyporeactivity after hemorrhagic shock was not the same in the 4 vascular beds examined. The loss of vascular reactivity to NE in the celiac (CA) and left femoral arteries (LFA) was more severe than in the superior mesenteric artery (SMA) and left renal artery (LRA), and NO and ET-1 inhibition improved the response to NE [3]. However, the mechanism(s) involved remain to be elucidated. The present study tested the hypotheses that the vascular bed diversity in vascular hyporeactivity to NE induced by hemorrhagic shock was associated with differential gene expression of iNOS, eNOS, IL-1β, IL-6, TNF-α, and ET-1 in the corresponding organs, and that NO or ET inhibition would improve vascular reactivity by down-regulating the gene expression of these factors.

TABLE 2

Changes in Vascular Reactivity in Superior Mesenteric Artery (SMA), Left Renal Artery (LRA), Celiac Artery (CA) and Left Femoral Artery (LFA) to Norepinephrine (NE, 3 μg/kg, iv) following Hemorrhagic Shock and the Effect of L-NAME and PD142893

	Baseline	End of hemorrhage	Post-shock 1h	Post-shock 2h	Post-shock 4h
SMA					
Hemorrhage	100	59.1 ± 12.5	44.2 ± 16.1	30.1 ± 13.5	13.9 ± 14.7
L-NAME group	100	82.5 ± 8.1	89.8 ± 9.3	72.3 ± 8.4	64.4 ± 13.1
PD142893 group	100	70.3 ± 20.8	80.3 ± 14.1	79.3 ± 16.8	65.5 ± 11.3
LRA					
Hemorrhage	100	56.7 ± 7.59	35.1 ± 9.28	16.6 ± 5.89	8.60 ± 5.84
L-NAME group	100	83.4 ± 6.18	88.5 ± 8.94	71.9 ± 17.8	69.4 ± 12.8
PD142893 group	100	76.6 ± 6.03	80.5 ± 12.0	76.1 ± 9.54	72.9 ± 16.6
CA					
Hemorrhage	100	43.2 ± 12.6†‡	31.6 ± 13.7	12.0 ± 8.05	2.98 ± 13.47
L-NAME group	100	70.1 ± 25.2	71.4 ± 21.2	58.7 ± 12.6	42.9 ± 6.64†‡
PD142893 group	100	67.9 ± 5.63‡	68.7 ± 7.79‡	70.8 ± 9.21	57.1 ± 18.5
LFA					
Hemorrhage	100	30.1 ± 11.9†‡	23.4 ± 7.26†	10.8 ± 9.03†	1.42 ± 3.47†‡
L-NAME group	100	67.4 ± 7.16†‡	60.4 ± 12.0†‡	51.3 ± 7.09†‡	38.4 ± 14.1†‡
PD142893 group	100	68.3 ± 6.19‡	62.5 ± 13.5†‡	68.3 ± 4.77*	54.0 ± 11.2*‡

Note. The vascular reactivity to NE at baseline was taken as the 100% response. n = 7 at each timepoint. * $P < 0.05$, as compared to L-NAME group; † $P < 0.05$, as compared to SMA; ‡ $P < 0.05$, as compared to LRA. All values in hemorrhage group were significantly less than baseline ($P < 0.05$). Except for the end of hemorrhage values in the PD142893 group for the SMA, all values in the L-NAME and PD142893 groups are significantly greater than corresponding values in the hemorrhage group ($P < 0.05$).

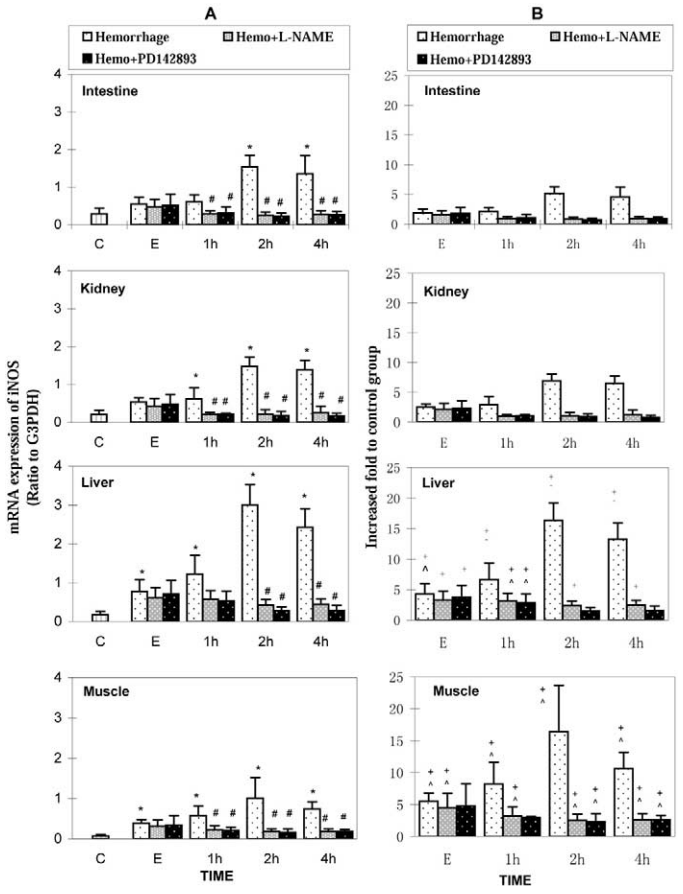

FIG. 1. mRNA expression of iNOS in intestine (ileum), left kidney, liver, and skeletal muscle after hemorrhage in the rat with or without L-NAME or PD142893 pre-treatment. (A) The ratio of iNOS mRNA expression to GAPDH. (B) The increased fold of iNOS mRNA expression to control group (pre-hemorrhage). (C) Control (pre-hemorrhage); (E) The end of the hypotensive period. $n = 7$ at each time point. iNOS mRNA expression was determined with RT-PCR. Data represent mean ± SD. $^{*}P < 0.05$ as compared to control group (pre-hemorrhage); $\#P < 0.05$ as compared to hemorrhage group; $+P < 0.05$ as compared to intestine, $^{\wedge}P < 0.05$ as compared to left kidney.

MATERIALS AND METHODS

Animals and Surgery

This study was approved by the Research Council and by the Animal Care and Use Committee of the U.S. Army Institute of Surgical Research. The experiments were performed in adherence to the National Institute of Health Guidelines on the Use of Laboratory Animals. Ninety-one male Sprague Dawley rats (Harlan, Indianapolis, IN), weighing 406 ± 44 g, were fasted 12 h but allowed water *ad libitum* before the experiment. On the day of experiment, rats were first anesthetized with 2 to 3% isoflurane and the right femoral artery and vein and right carotid artery were catheterized with PE tubing for monitoring the mean arterial blood pressure (MAP), for administering norepinephrine (NE), L-NAME, PD142893 and for bleeding [3]. A laparotomy was performed and the SMA, CA, and LRA were located and isolated, and the flow probes were mounted as previously described [3]. Then a flow probe was placed around the LFA after it was exposed and isolated [3]. All probes were connected to a Transonic flowmeter (Transonic Systems Inc., Ithaca, NY) for monitoring the blood flow of these arteries. After instrumentation, rats were allowed to equilibrate for 20 to 30 min.

Experimental Protocol

All hemorrhage experiments were performed under urethane anesthesia (900 mg/kg, i.p.). Operated and instrumented rats were divided into three groups: (1) hemorrhage alone; (2) hemorrhage + NG-nitro-L-arginine methyl ester (L-NAME), an NO synthase inhibitor; and (3) hemorrhage + PD142893, an ET receptor antagonist. An additional group of rats served as non-hemorrhaged controls. Rats, except in the control group, were bled (within 10 min) from the right carotid catheter until the MAP dropped to 50 mmHg and then maintained at this level for 60 min by either withdrawing or infusing blood as needed. This degree of hemorrhage corresponded to about 20 ml/kg [3]. L-NAME (10 mg/kg, i.v.) or PD142893 (0.1 mg/kg, i.v.) was administered 15 min before the end of hypotensive period. Doses of these agents were selected from the literature as described [3]. Shed blood was reinfused at the end of hypotensive period for all groups after evaluation of vascular activity, and some groups of rats

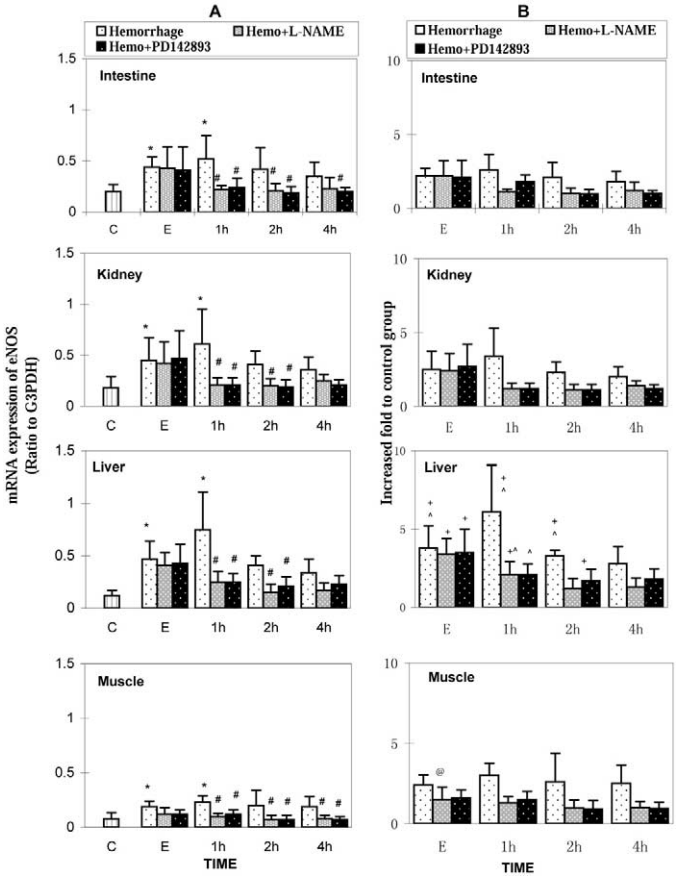

FIG. 2. mRNA expression of eNOS in intestine (ileum), left kidney, liver, and skeletal muscle after hemorrhage in the rat with or without L-NAME or PD142893 pre-treatment. (A) The ratio of eNOS mRNA expression to GAPDH. (B) The increased fold of eNOS mRNA expression to control group (pre-hemorrhage). (C) Control (pre-hemorrhage); (E) The end of the hypotensive period. $n = 7$ at each time point. eNOS mRNA expression was determined with RT-PCR. Data represent mean ± SD. $^{*}P < 0.05$ as compared to control group (pre-hemorrhage); $\#P < 0.05$ as compared to hemorrhage group; $+P < 0.05$ as compared to intestine; $^{\wedge}P < 0.05$ as compared to left kidney; $@P < 0.05$ as compared to liver.

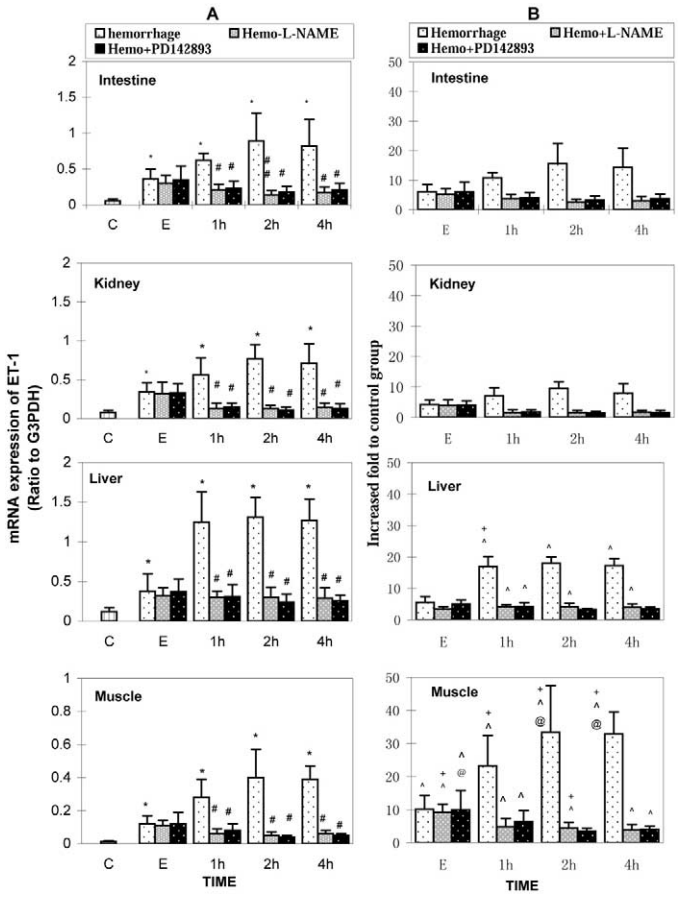

FIG. 3. mRNA expression of ET-1 in intestine (ileum), left kidney, liver, and skeletal muscle after hemorrhage in the rat with or without L-NAME or PD142893 pre-treatment. (A) The ratio of ET-1 mRNA expression to GAPDH. (B) The increased fold of ET-1 mRNA expression to control group (pre-hemorrhage). (C) Control (pre-hemorrhage); (E) The end of the hypotensive period. $n = 7$ at each time point. ET-1 mRNA expression was determined with RT-PCR. Data represent mean ± SD. $^{*}P < 0.05$ as compared to control group (pre-hemorrhage); $\#P < 0.05$ as compared to hemorrhage group; $+P < 0.05$ as compared to intestine; $^{\wedge}P < 0.05$ as compared to left kidney; $@P < 0.05$ as compared to liver.

were allowed to survive an additional 1, 2, or 4 h. All animals (n = 7/group/time period) were euthanatized with a pentobarbital-based euthanasia solution (Sleepaway; 2 ml, i.v.; Fort Dodge, IA) administered through the femoral vein catheter and intestine (ileum), liver, left kidney, and skeletal muscle from the left leg were collected and frozen at −75°C in RNAlater (Ambion RNA Co., Austin, TX) for the detection of inducible nitric oxide synthase (iNOS), endothelial NOS (eNOS), IL-1β, IL-6, TNF-α, and endothelin-1 (ET-1) gene expression.

Vascular reactivity to a vasoconstrictor was determined at each time point as the relative change in blood flow through the 4 arteries before and after norepinephrine (NE; 3 μg/kg bolus i.v. injection) administration. The results of this calculation at baseline were considered 100% and data at the other time points were expressed as a percentage of the baseline change. Other details regarding the determination of vascular reactivity have been described [3].

Total RNA Isolation

Total RNA was isolated from collected tissues using TRI Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. The RNA yield was determined spectrophotometrically with a SpectraMAX 250 (Molecular Devices, Sunnyvale, CA). The quality of the RNA was based on absorbance at 260 and 280 nm and gel electrophoresis on 0.9% agarose containing 1:10,000 SYBR Gold Nucleic Acid Stain (Molecular Probes, Portland, OR). Only undegraded RNA free of genomic DNA contamination was used. Isolated RNA was heated to 60°C for 5 min, quickly frozen on ice and preserved at −75°C for RT-PCR.

Real-Time Quantitative RT-PCR

One microgram of total RNA was reverse-transcribed to cDNA with Superscript II reverse transcriptase and poly dT priming according to the manufacturer's instruction (Life Technologies, Inc., Rockville, MD). Real-time quantitative PCR was performed with a LightCycler thermal cycler (Idaho Technology, Salt Lake City, UT) in a total reaction volume of 8 μl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.0–3.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 mg BSA, 0.5 μM sense and antisense primers, 1 μl of 1:3000 SYBER Green I (Molecular Probes), 1 μl of cDNA solution from reverse transcription, 0.36 U of KlenTaq DNA polymerase (AB Peptides, St. Louis, MO) and

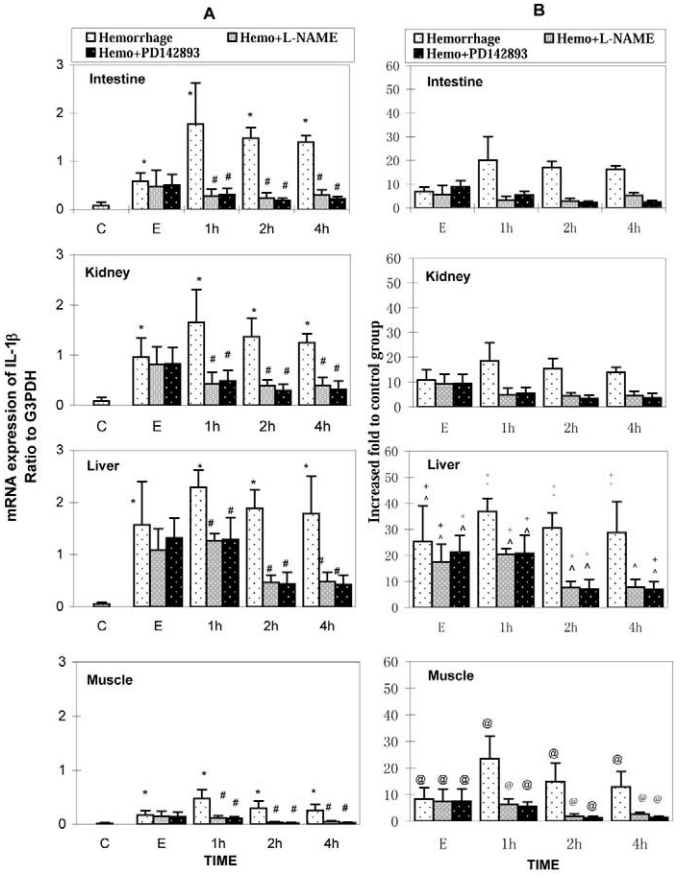

FIG. 4. mRNA expression of IL-1β in intestine (ileum), left kidney, liver, and skeletal muscle after hemorrhage in the rat with or without L-NAME or PD142893 pre-treatment. (A) The ratio of IL-1βmRNA expression to GAPDH. (B) The increased fold of IL-1βmRNA expression to control group (pre-hemorrhage). (C) Control (pre-hemorrhage); (E) The end of the hypotensive period. $n = 7$ at each time point. IL-1βmRNA expression was determined with RT-PCR. Data represent mean ± SD. $^{*}P < 0.05$ as compared to control group (pre-hemorrhage); $\#P < 0.05$ as compared to hemorrhage group; $+P < 0.05$ as compared to intestine; $^{\wedge}P < 0.05$ as compared to left kidney; $@P < 0.05$ as compared to liver.

0.064 μM TaqStart Antibody (Clontech, Palo Alto, CA). The sequences of specific primers were from previous reports (Table 1) [1, 18–22]. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal standard. The conditions of the PCR reactions varied for different primer sets and were determined empirically for each primer pair. For all PCR amplifications, the sample was preheated at 94°C for 15 s and received 30 to 45 cycles of 0 s at 94°C, 0 s at 55 to 58°C and 10 to 30 s at 72 to 74°C for a final elongation (Table 1). The melting curve was generated by gradually increasing temperature from 74°C to 96°C at 0.1°C/s. Fluorescence from reaction cuvettes was read at the end of each cycle and continuously during the melting curve step. Quantification of cDNA samples were achieved by reference to a standard curve generated from a series of dilution of positive control DNA with known copy number. Positive control DNA for each gene was generated by two rounds of PCR and gel purification and quantified by reference to quantitative DNA maker (BRL-Life Technologies, Inc.) on 2% agarose gel. The relative amounts of mRNA levels of the enzymes and mediators measured were calculated as the ratio to GAPDH expression.

Statistical Analysis

All data are presented as mean ± SD of n observations. Data analysis used SPSS v. 10.1 (SPSS Inc., Chicago, IL). Statistical differences of the changes in vascular responses to NE and gene expression were assessed by a three factor ANOVA (group, region, and time), followed by *post-hoc* Tukey test corrected for multiple comparisons. Correlations were run to determine Pearson correlation coefficients between vascular responsiveness and gene expression. A P value <0.05 was considered statistically significant.

RESULTS

Changes in Vascular Reactivity of SMA, LRA, CA, and LFA

Vascular reactivity to NE in the four observed arteries was decreased significantly ($P < 0.01$) after hemorrhagic shock (Table 2). SMA reactivity was reduced 40.9, 55.8, 69.9, and 86.1% at the end of the hypotensive period (E) and 1, 2, and 4 h later, respectively, compared with baseline. LRA reactivity was reduced 43.3, 64.9, 83.4, and 91.4%, respectively, during these times, while CA reactivity was reduced 56.8, 68.4, 88.0, and 97.0%, respectively, and LFA reactivity was reduced 69.9, 76.6, 89.2,, and 98.6%, respectively. In addition, the rate and magnitude of the loss of vascular responsiveness to NE among the vasculatures examined were not the same. For example, vascular responsiveness to NE in the CA and LFA decreased more severely and more rapidly ($P < 0.05$) than in the SMA and LRA (Table 2). L-NAME or PD142893 pretreatment attenuated the loss in vascular reactivity after hemorrhage significantly, and SMA and LRA vascular reactivity in these groups generally was better than in the CA and LFA.

Gene Expression in Ileum, Kidney, Liver, and Skeletal Muscle

To elucidate potential mechanisms to account for the hyporesponsiveness to NE and the regional diversity of vascular reactivity induced by hemorrhage, gene expression of iNOS, eNOS, ET-1, and select cytokines (IL-1β, IL-6, and TNF-α) was determined in the corresponding organs of SMA, LRA, CA, and LFA. Gene expression of GAPDH, as a housekeeping gene, was not affected significantly by the hemorrhage protocol. The results showed that mRNA levels of all enzymes and mediators, normalized to GAPDH expression, were increased significantly at the end of the hypotensive period in comparison to baseline levels, and peaked thereafter ($P < 0.05$; Figs. 1A through Fig. 6A). For example, iNOS mRNA expression began to increase at the end of hypotensive period in liver and skeletal muscle and peaked at 2 h in all organs examined (Fig. 1), whereas eNOS m RNA levels peaked at 1 h in all organs examined (Fig. 2). ET-1 mRNA levels in all organs examined were higher than baseline at the end hypotensive period and peaked at 1 h in liver and at 2 h in ileum, kidney, and skeletal muscle (Fig. 3). IL-1β, IL-6, and TNF-α mRNA levels peaked

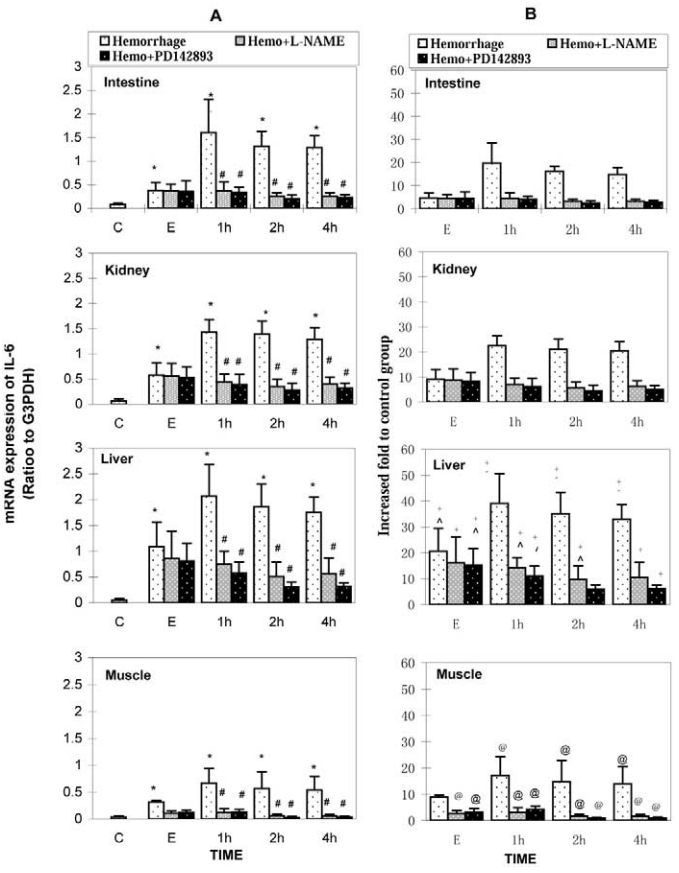

FIG. 5. mRNA expression of IL-6 in intestine (ileum), left kidney, liver, and skeletal muscle after hemorrhage in the rat with or without L-NAME or PD142893 pre-treatment. (A) The ratio of IL-6mRNA expression to GAPDH. (B) The increased fold of IL-6 mRNA expression to control group (pre-hemorrhage). (C) Control (pre-hemorrhage); (E) The end of the hypotensive period. $n = 7$ at each time point. IL-6 mRNA expression was determined with RT-PCR. Data represent mean ± SD. $^*P < 0.05$ as compared to control group (pre-hemorrhage); $^\#P < 0.05$ as compared to hemorrhage group; $^+P < 0.05$ as compared to intestine; $^\wedge P < 0.05$ as compared to left kidney; $^@P < 0.05$ as compared to liver.

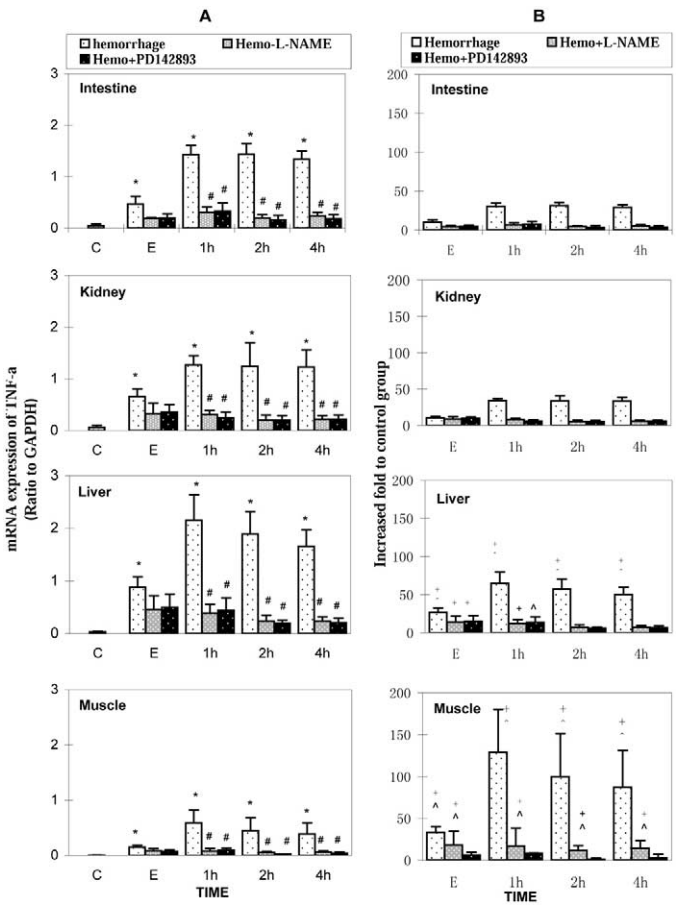

FIG. 6. mRNA expression of TNF-αin intestine (ileum), left kidney, liver, and skeletal muscle after hemorrhage in the rat with or without L-NAME or PD142893 pre-treatment. (A) The ratio of TNF-αmRNA expression to GAPDH. (B) The increased fold of TNF-αmRNA expression to control group (pre-hemorrhage). (C) Control (pre-hemorrhage); (E) The end of the hypotensive period. $n = 7$ at each time point. TNF-αmRNA expression was determined with RT-PCR. Data represent mean ± SD. *$P < 0.05$ as compared to control group (pre-hemorrhage); #$P < 0.05$ as compared to hemorrhage group; +$P < 0.05$ as compared to intestine; ^$P < 0.05$ as compared to left kidney.

at 1 h after the end of hypotensive period in all organs examined (Figs. 4 through 6).

When data were expressed relative to baseline levels, peak iNOS mRNA expression increased 5.2-, 6.9-, 16.4-, and 16.4-fold over pre-hemorrhage levels in ileum, left kidney, liver and skeletal muscle, respectively (Fig. 1B), and the fold increase in liver and skeletal muscle was significantly higher than in ileum and left kidney ($P < 0.05$; Fig. 1B). Peak eNOS mRNA levels were increased 2.6-, 3.4-, 6.1-, and 3.0-fold in ileum, left kidney, liver, and skeletal muscle, respectively (Fig. 2B). Peak ET-1 mRNA levels were increased 15.6-, 9.5-, 18.0-, and 33.4-fold in ileum, kidney, liver, and skeletal muscle, respectively (Fig. 3B). Peak IL-1β mRNA levels were increased 20.1-, 18.5-, 36.9-, and 23.5-fold in these organs, respectively (Fig. 4B). Peak levels of IL-6 mRNA were similar to those observed for IL-1β (Fig. 5B). Of the cytokines, TNF-α gene expression showed the highest levels with peak increases of 30.7-, 34.1-, 64.8-, and 129-fold, in ileum, kidney, liver, and skeletal muscle, respectively (Fig. 6B). In addition, the fold-increase of ET-1 and TNF-α mRNA expression in liver and skeletal muscle was significantly higher than in ileum and left kidney, and the fold increase in mRNA levels of the other cytokines was highest in liver than the other tissues.

L-NAME or PD142893 pre-treatment significantly reduced the up-regulation of mRNA levels of iNOS, eNOS, ET-1, and the cytokines observed in all tissues examined compared with the untreated hemorrhagic shock group (Figs. 1–6). In many cases, NOS and ET-1 mRNA levels in the L-NAME or PD142893 groups were at or near baseline values (Figs. 1–3). These inhibitors also significantly down-regulated the cytokine mRNA response to hemorrhage in all tissues examined (Figs. 4–6).

Correlation of Vascular Reactivity and Gene Expression

Correlation analyses were performed to determine any relationship between the increase in gene expression and the hyporeactivity observed after hemorrhage. As shown in Table 3A, the increase in gene expression for each variable measured correlated negatively with the decreased vascular reactivity of the corresponding vasculatures ($P < 0.01$). Correlation analyses were also performed for the L-NAME and PD142893 groups. It was observed that the better vascular reactivity in these groups compared to the untreated hemorrhage group, also correlated negatively ($P < 0.01$) with the gene expression of the various mediators in the corresponding organs (Table 3B).

DISCUSSION

Although vascular hyporeactivity following various kinds of shock has been well documented [1–3, 5, 6], many of these studies only investigated overall vascular reactivity based on the changes in MAP, or the

TABLE 3A

Correlation between the Changes in Vascular Reactivity of the Four Observed Arteries following Hemorrhagic Shock and mRNA Expression in the Corresponding Organs

	SMA (ileum)	LRA (kidney)	CA (liver)	LFA (muscle)
iNOS	−0.8608	−0.9049	−0.8716	−0.8705
eNOS	−0.5681	−0.5440	−0.4846	−0.8723
ET-1	−0.9586	−0.9833	−0.8823	−0.5577
IL-1β	−0.8394	−0.8668	−0.8647	−0.7133
IL-6	−0.8205	−0.9325	−0.9017	−0.7707
TNF-α	−0.8508	−0.7378	−0.8264	−0.7285

Note. All coefficients of correlation are significant, $P < 0.01$.

TABLE 3B

Correlation between the Improvement of Vascular Reactivity in the Four Arteries by L-NAME or PD142893 Pretreatment and Attenuation of mRNA Gene Expression in the Corresponding Organ

	SMA (ileum)		LRA (kidney)		CA (liver)		LFA (muscle)	
	L-NAME	PD142893	L-NAME	PD142893	L-NAME	PD142893	L-NAME	PD142893
INOS	−0.8533	−0.8709	−0.9091	−0.9110	−0.8491	−0.8803	−0.8326	0.8861
ENOS	−0.6059	−0.6665	−0.6435	−0.6305	−0.5506	−0.6235	−0.7469	−0.8413
ET-1	−0.9228	−0.9580	−0.9421	−0.9564	−0.8636	−0.8994	−0.6256	−0.6723
IL-1β	−0.8667	−0.8752	−0.8762	−0.8894	−0.7910	−0.8385	−0.7099	−0.7769
IL-6	−0.8501	−0.8598	−0.9329	−0.9503	−0.8768	−0.9040	−0.7568	−0.8150
TNF-α	−0.8717	−0.8736	−0.8306	−0.8421	−0.8158	−0.8625	−0.7527	−0.4947

Note. All coefficients of correlation are significant, $P < 0.01$.

responsiveness of a single blood vessel *in vitro* [1, 16]. In addition, little is known about the diversity of vascular hyporeactivity in different vascular beds after shock. Our previous work found that hemorrhagic shock-induced vascular hyporeactivity to NE was not the same in the four arteries examined [3]. Although the mechanism is not clear, we speculated that it might be related to the differential expression of cytokines, NO, or endothelin-1 in the corresponding organs.

The results from the present study indicated that the gene expression of iNOS, eNOS, IL-1β, IL-6, TNF-α, and endothelin-1 in the corresponding organs of select vasculatures increased significantly and the fold increase in mRNA levels in liver and skeletal muscle was higher than in intestine and left kidney. In addition, the increased mRNA levels were correlated negatively with the vascular hyporeactivity observed in the corresponding vasculatures. That is, the vascular responsiveness of CA and LFA was reduced more than the SMA and LRA, and gene expression of iNOS, eNOS, endothelin-1, and the select cytokines in liver and skeletal muscle, the tissues the CA and LFA supply, increased more than in intestine and left kidney, the organs the SMA and LRA supply. These findings supported our hypothesis that vascular hyporesponsiveness to NE and the vascular bed diversity observed following hemorrhagic shock were related closely to the differential expression of some cytokines, NOS and endothelin-1 in the corresponding organs. Previous studies also reported organ differences in the expression of some cytokines after shock. For example, Thiemermann *et al.* reported that iNOS activity in lung, liver, and spleen increased significantly after hemorrhagic shock, and iNOS activity in liver and spleen was significantly higher than that in mesentery and kidney [23]. ET-1, constitutive NOS (cNOS), TNF-α, and IL-1β reportedly also had differential expression in various organs during shock [24–26]. These latter studies, however, did not evaluate transcription regulation of these mediators with respect to vascular hyporeactivity.

In the present study, we were surprised by the increase in eNOS gene expression when normalized to GAPDH. As mentioned, GAPDH expression was not affected significantly by hemorrhage in our model and this observation agrees with other studies that used either smaller or larger hemorrhages than that used here [27, 28]. Although eNOS was first perceived to be expressed constitutively, recent evidence suggests that it is indeed, an inducible enzyme [29, 30], which may explain our present observations.

It is recognized that other factors can interfere with vascular reactivity after shock, and may account for some of the regional diversity of vascular hyporeactivity observed in the present study. Organs and tissues may develop different ischemic and hypoxic states during shock, leading to generation of oxygen free radical-induced lipid peroxidation, which has been implicated in the occurrence of vascular hyporeactivity [31–33]. In addition, some studies found that NO can affect different blood vessels differently, such that it has less modulating effect on vascular tone in resistance arteries than in conducting arteries or capacitance vessels [34]. These factors may be another reason for the regional diversity of vascular hyporeactivity observed.

Our results indicated that eNOS, IL-1β, IL-6, and TNF-αmRNA levels peaked earlier than iNOS and ET-1 in all organs examined. These results are consistent with previous reports [35, 36]. For example, Maass *et al.* reported that TNF-α began to increase 1 h post-burn injury in rat cardiac myocytes [35], and Collins *et al.* found iNOS mRNA expression detectable 60 min after hemorrhagic shock, and peak values at 150 min after shock [36]. This suggested that the acute phase cytokines such as TNF-α, IL-1β, and IL-6 may have an early role in the development of vascular hyporeactivity [37]. Further studies are necessary to determine whether the NOS and ET-1 gene expression is a later direct response to hemorrhagic shock or are induced by cytokines.

In the present study, pre-treatment with the non-selective NOS inhibitor, L-NAME, or non-selective endothelin receptor antagonist, PD142893, lessened the decrease in vascular reactivity associated with hemor-

rhage. This response may reflect a direct effect on NO and ET-1, but the current data suggest that improvement related to reduced up-regulation of gene expression of iNOS, eNOS, ET-1, IL-1β, IL-6, and TNF-α in the corresponding organs, may also be a factor. This concept was further supported by the statistically significant negative correlation observed between gene expression and vascular reactivity in the L-NAME and PD142893 groups. In addition, the similar results observed with NOS or ET-1 inhibition, illustrate the interaction between these mediators. Although NO and ET-1 reportedly have opposite effects on vascular tone, studies have shown that ET could induce the release of NO and NO may modulate ET receptor binding [3, 38]. Additional studies with these inhibitors are necessary to further elucidate the mechanisms involved.

It should be noted that the gene expression of cytokines determined in the present study only reflected the expression in tissues. We did not determine directly the expression in the blood vessels examined. In addition, the actual levels of the gene products were not measured, so it remains unknown whether the increased mRNA levels observed after hemorrhage are associated with higher production of these enzymes and mediators. Further study is warranted.

Nevertheless, the data from the present study suggest that the differential gene expression of NOS, ET-1, and select cytokines occurs at a time consistent with the development of vascular hyporesponsiveness to NE after hemorrhagic shock, and that NO or ET-1 inhibition reduces this effect not only through NOS and ET-1 inhibition, but through inhibition in the gene expression of inflammatory cytokines. In addition, the data suggest that the differential gene expression observed among the tissues examined, may account, at least in part, for vascular bed diversity observed in the development of vascular hyporeactivity after hemorrhagic shock.

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REFERENCES

1. St. John, J., Barbee, R. W., Sonin, N., Clemens, M. G., and Watts, J. A. Inhibition of poly(ADP-ribose) synthetase improves vascular contractile response following trauma-hemorrhage resuscitation. *Shock* **12:** 188, 1999.
2. Yaghi, A., Paterson, N. E., and McCormack, D. G. Vascular reactivity in sepsis: Importance of control and role of nitric oxide. *Am. J. Respir. Crit. Care Med.* **151:** 706, 1995.
3. Liu, L. M., Ward, J. A., and Dubick, M. A. Hemorrhagic shock induced vascular hyporeactivity to norepinephrine in select vasculatures of rats and the roles of nitric oxide and endothelin. *Shock* **19:** 208, 2003.
4. Kai, L., Wang, Z. F., Hu, D. Y., Yu, L. S., and Liu, L. M. Opioid receptor antagonists modulate Ca2+-activated K+ channels in mesenteric arterial smooth muscle cells of rats in hemorrhagic shock. *Shock* **19:** 85, 2003.
5. Kai, L., Wang, Z. F., Hu, D. Y., Yu, S. L., and Liu, L. M. Modulation of Ca2+ by opioid receptor antagonists in mesenteric arterial smooth muscle cells of rats in hemorrhagic shock. *J. Cardiovasc. Pharmacol.* **40:** 618, 2002.
6. Hasan, A., and McDonough, K. H. The effects of *Escherichia coli* sepsis and short-term ischemia on coronary vascular reactivity and myocardial function. *Shock* **8:** 305, 1997.
7. Li, S., Fan, S. X., and McKenna, T. M. Role of nitric oxide in sepsis-induced hyporeactivity in isolated rat lungs. *Shock* **5:** 122, 1996.
8. Pleiner, J., Heere-Ress, E., Langenberger, H., *et al.* Adrenoceptor hyporeactivity is responsible for Echerichia endotoxin-induced acute vascular dysfunction in humans. *Arterioscler. Thromb. Vasc. Biol.* **22:** 95, 2002.
9. Kamata, K., and Makino, A. A comparative study on the rat aorta and mesenteric arterial bed of the possible role of nitric oxide in the desensitization of the vasoconstrictor response to an alpha-1 adrenoceptor agonist. *Br. J. Pharmacol.* **120:** 1221, 1997.
10. Sato, S., Suzuki, A., Nakajima, Y., Iwamoto, T., Bito, H., and Miyabe, M. S-nitroso-N-acetylpenicillamine (SNAP) during hemorrhagic shock improves mortality as a result of recovery from vascular hyporeactivity. *Anesth. Analg.* **90:** 362, 2000.
11. Landín, L., Lorente, J. A., Renes, E., Canas, P., Jorge, P., and Liste, D. Inhibition of nitric oxide synthesis improves the vasoconstrictive effect of noradrenaline in sepsis. *Chest* **106:** 250, 1994.
12. Boyle, W. A., Parvathaneni, L. S., Bourlier, V., Sauter, C., Laubach, V. E., and Cobb, J. P. iNOS gene expression modulates microvascular responsiveness in endotoxin-challenged mice. *Circ. Res.* **87:** e18, 2000.
13. Kavuklu, B., Iskit, A. B., Guc, M. O., Ilhan, M., and Sayek, I. Aminoguanidine attenuates endotoxin-induced mesenteric vascular hyporeactivity. *Br. J. Surg.* **87:** 448, 2000.
14. Armstead, W. M. Opioids and nitric oxide contribute to hypoxia-induced pial arterial vasodilation in newborn pigs. *Am. J. Physiol.* **268:** H226, 1995.
15. Simper, D., Strobel, W. M., Linder, L., and Haefeli, W. E. Indirect evidence for stimulation of nitric oxide release by tumor necrosis factor-alpha in human veins in vivo. *Cardiovasc. Res.* **30:** 960, 1995.
16. Zingarelli, B., Squadrito, F., Altavilla, D., Calapai, G., Di Rosa, M., and Caputi, A. P. Role of tumor necrosis factor-alpha in acute hypovolemic hemorrhagic shock in rats. *Am. J. Physiol.* **266:** H1512, 1994 .
17. Robert, R., Chapelain, B., Jean, T., and Néliat G. Interleukin-1 impairs both vascular contraction and relaxation in rabbit isolated aorta. *Biochem. Biophys. Res. Commun.* **182:** 733, 1992.
18. Zhao, B., Bowden, R. A., Stavchansky, S. A., and Bowman, P. D. Human endothelial cell response to gram-negative lipopolysaccharide assessed with cDNA microarrays. *Am. J. Physiol.* **281:** C1587, 2001.
19. Sergio, A., Pang, Y., Romero, S., *et al.* Nitric oxide and vessel

caliber: Inducible nitric oxide synthase and the regulation of central vessel caliber in the fetal rat. *Circulation* **94:** 1948, 1996.

20. Ogle, C. K., Kong, F., Guo, X., Wells, D. A., Aosasas, N. G., and Horseman, N. The effect of burn injury on suppressors of cytokine signaling. *Shock* **14:** 392, 2000.
21. Szaflarski, J., Burtrum, D., and Silerstein, F. S. Cerebral hypoxia-ischemia stimulates cytokine gene expression in perinatal rats. *Stroke* **26:** 1093, 1995.
22. Mattson, D. L., and Wu, F. Nitric oxide synthase activity and isoforms in rat renal vasculature. *Hypertension* **35:** 337, 2000.
23. Thiemermann, C., Szabó, C., Mitchell, J. A., and Vane, J. R. Vascular hyporeactivity to vasoconstrictor agents and hemodynamic decompensation in hemorrhagic shock is mediated by nitric oxide. *Proc. Natl. Acad. Sci.* **90:** 267, 1993.
24. Minchenko, A. G., Armstead, V. E., Opentanova, I. L., and Leffer, A. M. Endothelin-1, endothelin receptors and ecNOS gene transcription in vital organs during traumatic shock in rats. *Endothelium* **6:** 303, 1999.
25. Sharma, A. C., Singh, G., and Gulati, A. Decompensation characterized by decreased perfusion of the heart and brain during hemorrhagic shock: Role of endothelin-1. *J. Trauma* **53:** 531, 2002.
26. Rajnik, M., Sakowski, C. A., Thomas, K. E., Li, Y. Y., Rollwagen, F., and Vogel, S. Induction of early inflammatory gene expression in a murine model of nonresuscitated, fixed-volume hemorrhage. *Shock* **17:** 322, 2002.
27. Li, Q., Goodchild, A. K., and Pilowsky, P. M. Effect of hemorrhage on the expression of neurotransmitter-related genes in rat ventrolateral medulla: a quantitative real-time RT-PCR study. *Molec. Brain Res.* **114:** 46, 2003.
28. Alam, H. B., Stegalkina, S., Rhee, P., and Koustova, E. cDNA array analysis of gene expression following hemorrhagic shock and resuscitation in rats. *Resuscitation* **54:** 195, 2002.
29. Bobadilla, N. A., Tapia, E., Jimenez, F., *et al.* Dexamethasone increases eNOS gene expression and prevents renal vasoconstriction induced by cyclosporin. *Am. J. Physiol.* **277:** F464, 1999.
30. Cirino, G., Fiorucci, S., and Sessa, W. C. Endothelial nitric oxide synthase: The Cinderella of inflammation. *Trends in Pharmacol. Sci.* **24:** 91, 2003.
31. Macarthur, H., Westfall, T. C., Riley, D. P., Misko, T. P., and Salvemini, D. Inactivation of catecholamines by superoxide gives new insights on the pathogenesis of septic shock. *Proc. Natl. Acad. Sci.* **97:** 9753, 2000.
32. Garrison, R. N., and Cryer, H. M. Role of the microcirculation to skeletal muscle during shock. *Prog. Clin. Biol. Res.* **299:** 43, 1989.
33. Katusic, Z. S. Superoxide anion and endothelial regulation of arterial tone. *Free Rad. Biol. Med.* **20:** 443, 1996.
34. Smith, K. M., Macmillan, J. B., McCulloch, K. M., and McGrath, J. C. NOS inhibition potentiates norepinephrine but not sympathetic nerve-mediated co-transmission in resistance arteries. *Cardiovasc. Res.* **43:** 762, 1999.
35. Maass, D. L., Hybki, D. P., White, J., and Horton, J. W. The time course of cardiac NF-kB activation and TNF-α secretion by cardiac myocytes after burn injury: Contribution to burn-related cardiac contractile dysfunction. *Shock* **17:** 293, 2002.
36. Collins, J. L., Vodovotz, Y., Hierholzer, C., *et al.* Characterization of the expression of inducible nitric oxide synthase in rat and human liver during hemorrhagic shock. *Shock* **19:** 117, 2003.
37. Szabo, C., and Thiemermann, C. Invited opinion: Role of nitric oxide in hemorrhagic, traumatic, and anaphylactic shock and thermal injury. *Shock* **2:** 145, 1994.
38. Warner, T.D. Relationship between the endothelin and nitric oxide pathways. *Clin. Exp. Pharmacol. Physiol.* **26:** 247, 1999.